Research Article

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Synthesis and Investigation of Cytotoxicity in Different Cell Lines of Novel Hydroxypyranones

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ABSTRACT

Mannich bases synthesized from kojic acid have been determined in extensive studies conducted by our study group that they have a wide variety of biological activities. In this study, new anticancer-active compounds were synthesized from compounds with high anticancer activity. The compounds' structures were identified through the utilization of spectroscopic methods and elemental analyses. The cytotoxicity screening of the compounds were done by MTT assay on different human cancer cell lines including MCF-7 and MDA-MB-231 (human breast cancer), SK-MEL (human melanoma), HT-29 (human colon adenocarcinoma), A549 (human lung carcinoma) cell line.Compound 1 bearing iodomethyl moiety, showed remarkable cytotoxicity with IC50 values in the range of 8.11 and 21.24 µg/mL. The IC50 value on Vero (African green monkey kidney epithelial) is over 100 µg/mL which means low cytotoxicity against healthy cells. Compared to this, SK-MEL cells have 12 times less IC50 value, with a therapeutic index over 12. The closest cytotoxic effectivity to KOJI MG84 which is previously reported as a cytotoxic agent, is observed on the SK-MEL cell line in compound 1, with 4 times less cytotoxic activity.

Keywords: kojic acid, Mannich bases, cytotoxicity, anticancer

1. Introduction

Cancer, which is considered one of the most important health problems of our age due to its high incidence and lethality, is increasing in incidence due to increasing population and exposure to carcinogenic substances. It is a leading cause of death accounting for approximately 10 million deaths in 2020 and escalating public health. It is defined by unchecked cell growth, metastasis and the invasion of tissues, ranking as the second most common cause of death globally, following cardiovascular disease [1, 2].

Cancer is fundamentally associated with genes because it originates from abnormalities in the DNA sequence, and since genes govern the division and regulation of cells [2]. The unregulated growth and proliferation of unusual cells in the body defines the complex group of disorders known as cancer. Through metastasis, these cells have the ability to penetrate nearby tissues and, in some cases, move to other areas of the body.

Based to the Global Burden of Disease (GBD) study, colorectal, lung, and breast cancer were found to be the most prevalent cancers in women, while prostate, colorectal, and lung cancer were most common among men. Breast and cervical cancer are the most common cancers globally among people aged 15-39, a demographic where early detection and cancer prevention are critical. Leukaemia and liver cancer are the common causes of cancer-related deaths in this age group [3]. Cancer treatment has primarily aimed at restoring proper regulation of these cellular processes. There have been numerous clinical studies conducted to improve cancer treatments, such as surgery, radiation therapy, chemotherapy, antibody therapies, and immunotherapy. While diverse treatment regimens are employed for different forms of cancer, the existing treatment approaches are often inadequate. Furthermore, numerous cancer types have a propensity to relapse and develop resistance following treatment [4].

Chemotherapy and Radiation treatments have significant side effects owing to their harmful impact on normal cells. Immunotherapy and antibody therapy can specifically aim at cancer cells; however, these approaches are costly and exhibit a restricted range of targets [5]. In order to minimise the side effects of anticancer drugs and to obtain more potent drugs, the development of new drug molecules that are selective and direct cells to apoptosis remains an important approach [6, 7].

In comprehensive studies conducted by our research group, it has been determined that Mannich bases synthesized from kojic acid having structure of hydroxypyranone ring, showed various biological activities such as antityrosinase [8], antibacterial [9], antiviral [10], anticonvulsant [11], antioxidant [12], and antiepileptic [13].

The cytotoxic effects of Mannich bases on cell viability were determined in our recent research through in vitro assays using Sulforhodamine B assay with A375 human melanoma, HGF-1 human gingival fibroblast, and MRC-5 lung epithelial cell lines [14].

It is known that kojic acid derivative Mannich bases have been hypothesized to be more effective agents compared to Vemurafenib, being used in the treatment of melanoma, as they demonstrate significant cytotoxic effects in cancer cells, do not disrupt the morphology of healthy cells, and suppress multidrug resistance by directing cells towards apoptosis instead of necrosis Based on the obtained findings, International Patent (PCT/TR2018/050724) and Turkish Patent (TR2017/20155) applications have been filed. Furthermore, it has been demonstrated that these compounds inhibit melanogenesis in A375 cell lines. [14, 15].

In another study, the anticancer activities of a kojic acid derivative were investigated in a liver hepatocellular carcinoma cell line (HEPG2), and it was determined that the selected most active compound exhibited its effects by activating the intrinsic apoptotic pathway and did not induce drug resistance [16]. Furthermore, derivatives synthesized from kojic acid showed promising cytotoxicity results in HEPG2 hepatocellular carcinoma, MDA-MB-231 and MCF-7 breast cancer cell lines. Additionally, the cell death efficacy in breast cancer cell lines was supported by caspase 8 and 9 analyses [17] (Figure 1).

Within the scope of this study, based on the aforementioned results, we have conducted research on the anticancer activity of Mannich bases in several cell lines and have achieved encouraging results for which patent applications have been made. The synthesis of new compounds with modifications in the chemical structures of Mannich bases, including previously unreported derivatives of the piperazine ring and isosteric derivatives, has been carried out. The compounds' structures were determined by ^{1}H- and

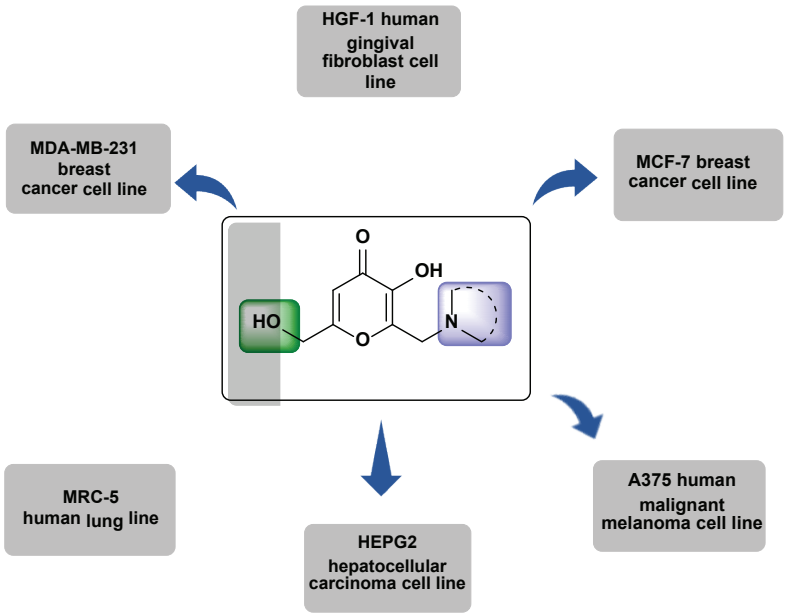

Figure 1. Cancer cell lines studied for cytotoxic Mannich bases generated from kojic acid.

^{13}C-NMR, and elementary analysis. Furthermore, their anti-cancer properties across five distinct tumor cells, including MCF-7 and MDA-MB-231 (human breast cancer), SK-MEL (human melanoma), HT-29 (human colon adenocarcinoma), A549 (human lung carcinoma) alongside a healthy cell line (Vero - green African monkey kidney epithelium) were assessed.

2. Material and Methods

2.1. Material

The chemical agents used in the synthesis, were commercially achieved from Merck and Aldrich Chemical Co. Melting points were measured using a Buchi M-560 Melting Point Apparatus and reported without any adjustments. NMR spectra were acquired using a Varian Mercury 500 MHz spectrophotometer in DMSO-d_6. Tetramethylsilane which has a chemical shift of, ppm, was employed as an internal reference to assure accuracy. A Micromass ZQ LC-MS and the electrospray ionization technique were used to create the mass spectra. HPLC was employed with Waters Alliance and C18 columns. We conducted elemental analyses were conducted in Faculty of Pharmacy, Central Laboratory, Ankara University, using a Leco CHNS-932 analyzer. The purity of the compounds were assessed by using TLC with Kieselgel 60 F_{254} chromatoplates from Merck®.

2.2. Methods

2.2.1. Chemistry

Synthesis procedure of the Mannich bases

After adding 37 % formalin (3 mmol) to the substituted secondary amine derivative, the mixture is stirred. To the solution, 3 mmol of kojic acid is added followed by 20 mL of methanol. The mixture was shaken vigorously for 20 min at 25°C. The precipitate is rinsed with ice-cold methanol following vacuum filtration. Finally, it is crystallized using appropriate solvents. (If the reaction does not proceed, adjust the pH to 8 using a 0.1 N NaOH solution) (Figure 2).

3-Hydroxy-6-(hydroxymethyl)-2-{[4-(4-iodophenyl) piperazin-1-yl]methyl}-4H-pyran-4-one (Compound 1)

Pale yellow solid; yield: 81.68%; mp: 190-191°C; ^{1}H-NMR: (500 MHz; DMSO$_{d6}$) δ ppm: 2.57 (4H; t; piperazine), 3.12 (4H; t; piperazine), 3.57 (2H; s; -CH_2-), 4.31 (2H; d; -CH_2OH), 5.69 (1H; t; -CH_2OH), 6.31 (1H; s; H5), 6.76 (2H; d; Ar-H'); 7.47 (2H; d; Ar-H'), 9.05 (1H; brs; -pyran3OH); ^{13}C-NMR (DMSO, 125 MHz) δ ppm: 48.11, 52.68, 53.97,

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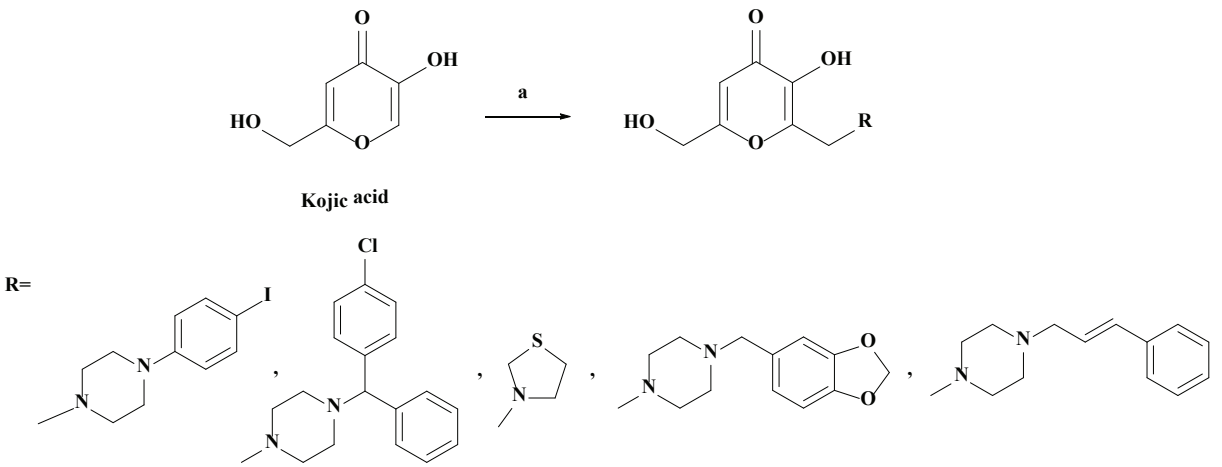

Figure 2. The synthetic route of compounds **1-5**. (a: substituted secondary amines, formalin, MeOH, rt)

60.07, 81.11, 109.42, 118.27, 137.72, 144.23, 146.00, 151.00, 168.12, 174.08; ESI-MS (m/z): 289 ((%100), 443 (%100, M^++H).

2-{[4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl]methyl}-3-hydroxy-6-(hydroxymethyl)-4H-pyran-4-one (Compound 2)

Yellow solid; yield: 67.58%; mp: 83-84°C; ^{1}H-NMR: (500 MHz; $DMSO_{d6}$) δ ppm: 2.16 (10H; brs; piperazine), 2.64 (10H; brs; piperazine), 3.51 (2H; s; -CH_2-), 4.33 (2H; d; -CH_2OH), 5.67 (H; brs; Ar-CH), 6.31 (1H; s; H5), 7.17 (9H; d; Ar-H'); 7.50 (2H; d; Ar-H'), 9.00 (1H; brs; -piran^{3}OH); ^{13}C-NMR (DMSO, 125 MHz) δ ppm: 51.71, 53.00, 53.95, 60.03, 74.40, 109.40, 127.47, 128.01, 129.00, 129.07, 129.82, 131.74, 142.38, 142.74, 144,15, 146.96, 168.06, 174.06; ESI-MS (m/z): 441 (%100, M^++H), 443 (%100, M^++H+2).

3-Hydroxy- 6-(hydroxymethyl)-2-(thiazolidin-3-ylmethyl)-4H-pyran-4-one (Compound 3)

Pale yellow solid; yield: 24.53%; mp: 134-135°C; ^{1}H-NMR: (500 MHz; $DMSO_{d6}$) δ ppm: 3.02 (2H; t; thiazolidine), 2.87 (2H; t; thiazolidine), 4.06 (2H; s; thiazolidine), 3.52 (2H; s; -CH_2-), 4.31 (2H; d; -CH_2OH), 5.70 (1H; t; -CH_2OH), 6.33 (1H; s; H^5), 9.08 (1H; brs; -pyran3OH); ^{13}C-NMR (DMSO, 125 MHz) δ ppm: 29.33, 48.78, 57.74, 60.01, 61.08, 109.40, 143.81, 147.52, 168.29, 174.22; ESI-MS (m/z): 155 (%100), 244 (M^++H).

2-{(4-[Benzo[d][1,3]dioxol-5-ylmethyl)piperazin-1-yl]methyl}-3-hydroxy-6-(hydroxymethyl)-4H-pyran-4-one (Compound 4) (Benzodioxole: BDX)

White solid; yield: 80%; mp: 203-204°C; ^{1}H-NMR: (500 MHz; $DMSO_{d6}$) δ ppm: 2.35 (6H; brs; piperazine), 2.51 (6H; brs; piperazine), 3.51 (2H; s; -CH_2-), 4.29 (2H; s; -CH_2OH), 6.31 (1H; s; H^5), 6.73 (1H; d; 1,3-BDX-H'); 6.83 (2H; m; 1,3-BDX-H'), 5.98 (2H; s; 1,3-BDX-H'), 6.83 (2H; m; 1,3- BDX-H'), 9.02 (1H; brs;-pyran^{3}OH), 3.36 (7H; brs; 1,3-BDX-CH_2); ^{13}C-NMR (DMSO, 125 MHz) δ ppm: 52.73, 52.87, 54.02, 60.04, 62.04, 101.22, 108.29, 109.40, 109.51, 122.41, 144.00, 144.12, 144.60, 147.64, 168.05, 174.04; ESI-MS (m/z): 375 (%100, M^++H).

2-[4-(3-Phenylprop-2-en-1-yl)piperazin-1-yl]methyl-3-hydroxy-6-(hydroxymethyl)-4H-pyran-4-one (Compound 5)

White solid; yield: 85%; mp: 185-187°C; ^{1}H-NMR: (500 MHz; $DMSO_{d6}$) δ ppm: 3.51 (2H; s; -CH_2-), 4.30 (2H; s; -CH_2OH), 5.68 (1H; brs; -CH_2O*H*), 6.25-6.32 (2H; m; H^5), 7.21-724 (1H; m; Ar-H'); 7.30-7.33 (2H; m; Ar-H'), 7.42-7.44 (2H; m; Ar-H'), 9.02 (1H; brs; -pyran^{3}OH), 3.07 (2H; d; -C3H4-), 6.25- 6.32 (2H; m; -C^{3}H^4-), 6.51 (2H; d; -C^{3}H^4-); ^{13}C-NMR (DMSO, 125 MHz) δ ppm: 52.96, 53.02, 54.05, 60.05, 60.55, 109.35, 126.65, 127.58, 127.84, 129.03, 132.40, 137.12, 144.12, 147.03, 168.05, 174.04; ESI-MS (m/z): 357 (%100, M^++H).

2.2.2. Cell Culture and Cytotoxic Activity

The cancer cells MCF-7 (human breast adenocarcinoma), MDA-MB–231 (human breast adenocarcinoma), SK-MEL (human melanoma carcinoma), A549 (human lung carcinoma), HT-29 (human colon adenocarcinoma) and healthy cell line Vero (African green monkey kidney epithelial) were produced from Izmir Katip Çelebi University, Faculty of Pharmacy, Izmir, Türkiye. Cells were cultured in media, RPMI 1640 or DMEM-Ham F12 in a humidified environment at 37°C with 5% CO_2.

The cytotoxic activity of compounds was analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test in both cancer and normal cell lines at concentrations of 6.25, 12.5, 25, 50, and 100 µg/mL, respectively. Cells were cultured in 96-well plates at equal numbers of 6000 cells/well per well and incubated for 24 hours before adding compounds, negative control 1 (cells + medium), negative control 2 (cells + medium + 1% DMSO), and positive control doxorubicin (10µg/ml) were added. Cells were incubated at 37°C for 72 h. The viability of the cells was determined. The media in each well was replaced with MTT and then placed in an incubator at 37°C and 5% CO_2 atmosphere. Absorbance was quantified in triplicate at 570-690 nm using a multimode microplate reader (BMG Labtech Clariostar Plus, Germany). The cytotoxicity of the compounds was expressed as cell viability percentage (Microsoft Excel) [18].

2.2.3. Statistical Analysis

The Student's t-test was used at a significance level of $p \leq 0.01$ to demonstrate strong significance and at a significance level of $p \leq 0.05$ to indicate statistical differences. The information was displayed in the form of average values with the SEM indicated. The mean values of three experiments were obtained, and presented with their corresponding standard deviations.

3. Results and Discussion

3.1. Synthesis

In the context of current study, we employed the procedure outlined in Figure 2 to synthesize five novel Mannich base derivatives. Kojic acid exhibits phenol-like characteristics, enabling their efficient aminomethylation at room temperature within a brief duration, resulting in high yields. This phenomenon takes place specifically in the Mannich reactions occurring *ortho* to the enolic -OH group. The Mannich reaction mechanism is based on increased reactivity in a basic environment attributed to increased electronegativity at position 6 of the hydroxypyron skeleton [14]. Compound 1-5 were synthesised by a high yield reaction at room temperature using benzylpiperazine derivatives, kojic acid and 37% formalin (Table 1) and characterized through the utilization of LC-MS, ^{1}H and ^{13}C NMR spectroscopic techniques.

Signal assignments were made by considering the chemical shifts and the pattern of signal intensities. The broad and singlet peaks observed in the majority of the compounds may be due to the presence of nitrogen atoms in the piperazine rings. In the ^{1}H NMR spectra, triplet peaks were observed in the range of 2.36 to 2.63 ppm, corresponding to the CH_2- group of the piperazine moiety. The chemical shift range of 6.76 to 7.50 ppm in the ^{1}H NMR detected the phenyl ring protons. The characteristic H^5 proton of the pyranone ring was identified by its singlet peaks, typically appearing at around 6.5 ppm. The ^{13}C-NMR signals closely matched the expected chemical structures, indicating a good conformity between the experimental data and the proposed molecular compositions. The carbonyl carbons of the pyranone ring were observed at approximately 168 ppm in the ^{13}C-NMR spectra. Consistent fragmentation patterns were found in the mass spectra for each compound.

3.2. Cytotoxic activity

Firstly, all compounds were evaluated for their effects on cell viability at the lowest dose studied. The most sensitive cell lines were analyzed. IC_{50} values were then calculated and compared with each other and with doxorubicin, a cytotoxic control compound used clinically in cancer treatment. The relationship between the chemical structures of the compounds and their biological activities was tried to be established and interpreted.

Compound 1 which is carrying iodine atom substituted at the para position of the phenyl ring, exhibited the lowest cell viability of 54.18% on the SK-MEL cell line, in the lowest concentration as 6.25 µg/mL. However, in the same concentration, the cell viability on the Vero-green African monkey kidney epithelial cell line, which represents healthy cells, was 100.68% (Figure 3). This suggests that at the

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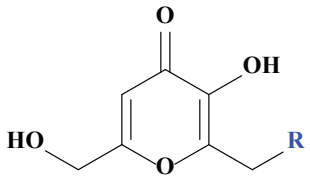
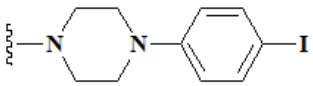
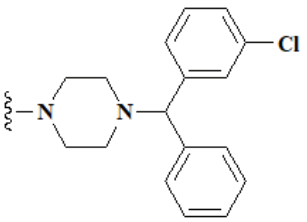
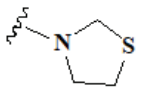
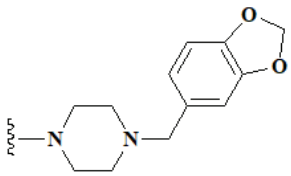
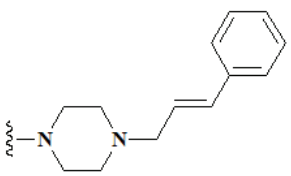
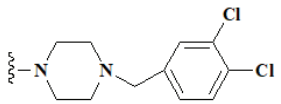
Table 1. The cytotoxicity of the synthesized compounds (IC_{50} values in µg/mL)



Comp.	R	Cells					
		IC_{50} (±S.D)					
		MDA-MB-231	A549	SK-MEL	HT-29	MCF-7	Vero
1		15.24 (±2.13)	13.31 (±3.11)	8.11 (±1.34)	21.24 (±5.33)	19.32 (±6.13)	>100
2		42.44 (±5.34)	23.31 (±5.21)	9.41 (±1.43)	11.13 (±2.23)	14.22 (±3.14)	>100
3		12.84 (±1.26)	18.43 (±3.56)	10.21 (±2.21)	19.34 (±2.41)	17.32 (±3.22)	98.21 (±7.43)
4		81.32 (±8.44)	43.24 (±4.12)	15.31 (±3.24)	38.42 (±3.41)	27.24 (±2.42)	>100
5		93.42 (±9.32)	62.24 (±6.33)	37.13 (±4.21)	54.21 (±5.24)	42.13 (±5.11)	>100

KOJIMG-84		6.891	3.324	2.245	5.342	-	12.214
DOX		1.09 ± 0.4	0.4 ± 0.02	2.6 ± 0.1	1.14 ± 0.2	1.14 ± 0.5	2.07 ± 0.7

DOX: Doxorubicin

same dose, this compound is cytotoxic against tumor cells but less cytotoxic against healthy cells.

As seen in Figure 4, for Compound 2, bearing benzydril moiety as the amine group of the Mannich base, the lowest concentration of 6.25 µg/mL resulted in 55.4198% cell viability on the SK-MEL cell line. However, in the same concentration, the cell viability on the Vero-green African monkey kidney epithelial cell line, which represents healthy cells, was 101.1933%.

Compound 3 exhibited the lowest cell viability of 55.2259% on the SK-MEL cell line at 6.25 µg/mL dose. However, in the same concentration, the cell viability on the Vero-green African monkey kidney epithelial cell line, which represents healthy cells, was 144.3204% Figure 5.

In Compound 4, the lowest cell viability of 58.3999% was observed on the SK-MEL cell line at concentration of 12.5 µg/mL. However, at the same concentration, the cell viability on the Vero-green African monkey kidney epithelial cell line, which represents healthy cells, was 105.057% Figure 6.

At 25 µg/mL dose of Compound 5, the SK-MEL cell line exhibited the lowest cell viability of 59.00486%. In contrast, the Vero cell line, representing healthy cells, displayed a cell viability of 108.4485% at the same concentration Figure 7.

When evaluated in terms of structure-action relationships, it can be said that piperazine groups and related halogen atoms are common in the structure of compounds 1 and 2, which are the 2 most active derivatives against SK-MEL cells. However, the benzhydryl group in compound 2 has a larger structure in terms of steric-hindrance since it contains 2 phenyl rings. In HT29 (IC_{50}: 11,13 µg/mL) and MCF-7 (IC_{50}:14,22 µg/mL) cell lines, this turned into an advantage as it had a lower IC_{50} value among all the compounds.

Among the new molecules synthesized, Compound 3, which contains a thiazolidine group as a secondary amine derivative different from piperazine, showed the highest cytotoxicity against SK-MEL cells. In addition, this compound was found to be the most effective compound in the series with an IC_{50} value of 12.84 µg/mL against MDA-MB-231 cells. Compound 4, which have benzodioxole group as a condensed ring system in its structure, showed a moderate effect in the series with IC_{50} of 15.31 µg/mL against SK-MEL cells, while this effect decreased against other cell lines. The weakest compound in the series in terms of cytotoxic activity is compound 5. Since this compound carries the *trans*-cinnamyl group in its structure, it can be interpreted that the activity is negatively affected by this group.

As a result, with the data obtained from this study, compounds more cytotoxic were synthesized against various cancer cells than healthy cells and their structures were elucidated. However, among the new derivatives, no derivative with a more potent activity than our patented compound has been identified. In further studies, it is planned to carry out studies in terms of its mechanism of action and interaction with the target site and to develop the activity through molecular modification.

4. Conclusions

Among the newly synthesized compounds, the highest cytotoxic activity (lowest IC_{50} value = 8.11 µg/mL) was observed in compound 1 which is bearing iodine atom as a substituent, against the SK-MEL cell line Figure 8. No significant cytotoxicity was observed in healthy cell line with IC_{50} of greater than 100 µg/mL.

Compared to the healthy cell line, SK-MEL cells exhibited an IC_{50} value that was 12 times lower, indicating a therapeutic index greater than 12.

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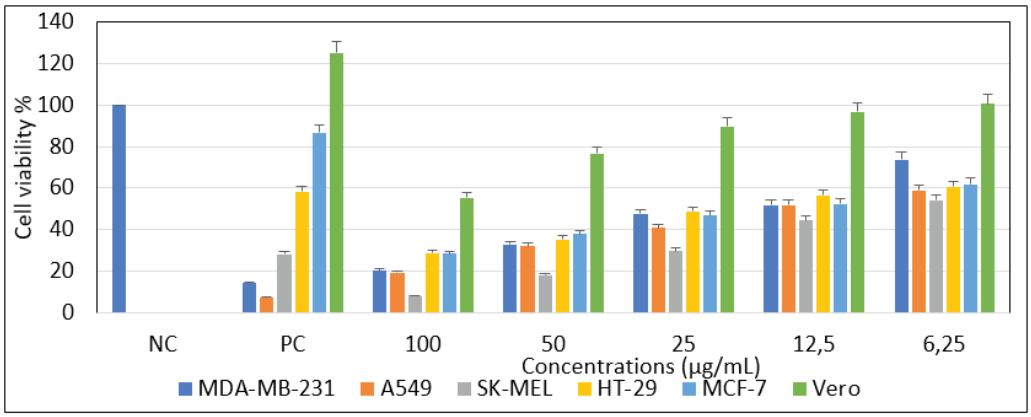

Figure 3. Graph of cell viability percentage and concentration for Compound 1 (NC: negative control, PC: positive control).

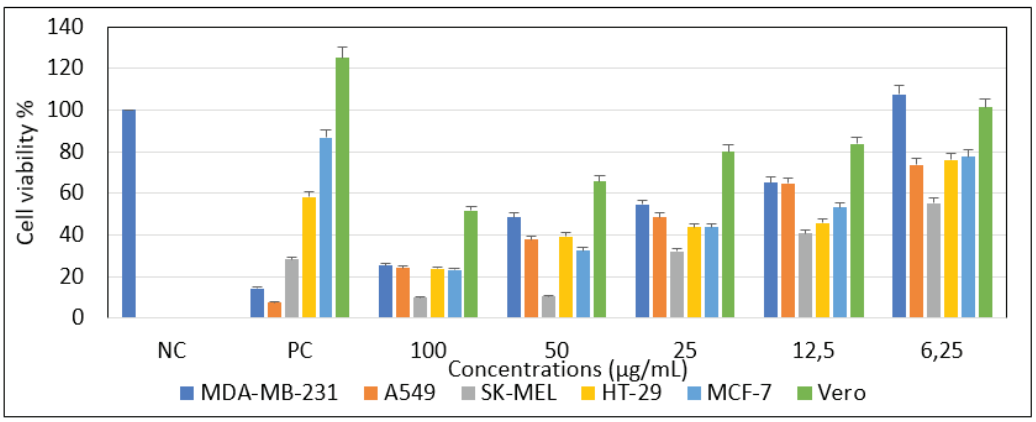

Figure 4. Graph of cell viability percentage and concentration for Compound 2 (NC: negative control, PC: positive control).

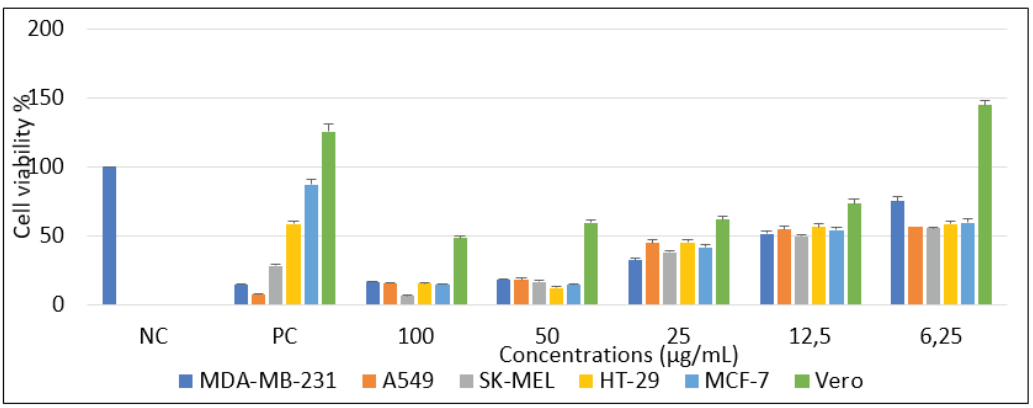

Figure 5. Graph of cell viability percentage and concentration for Compound 3 (NC: negative control, PC: positive control).

For all compounds, the SK-MEL cell line showed the lowest IC_{50} values, indicating its higher sensitivity compared to the other tested cell lines. As a general trend, we can say that this is followed by HT-29, A549, MCF-7 and MDA-MB-231 cell lines respectively. In fact, considering that these Mannich bases are known to be highly cytotoxic against malignant melanoma cells in our previous studies, the results

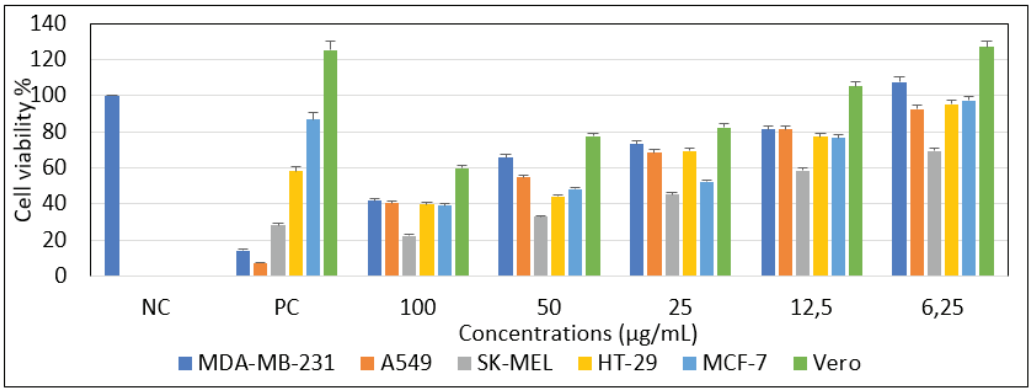

Figure 6. Graph of cell viability percentage and concentration for Compound 4 (NC: negative control, PC: positive control).

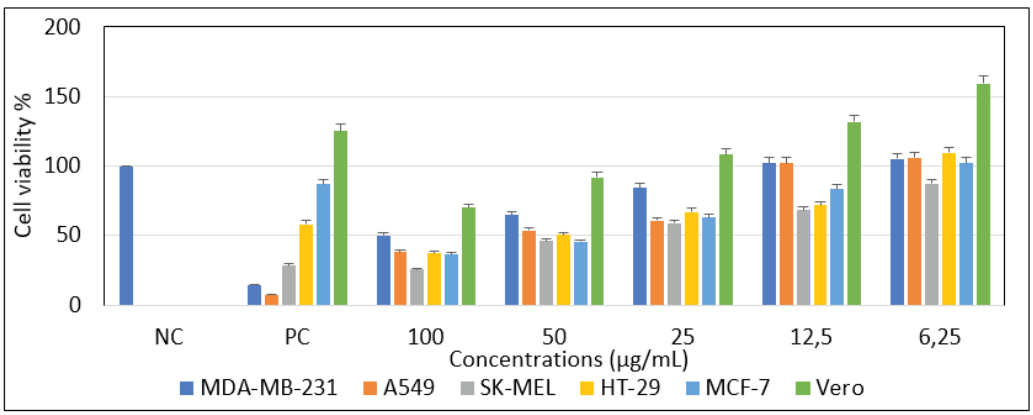

Figure 7. Graph of cell viability percentage and concentration for Compound 5 (NC: negative control, PC: positive control).

were as expected and in agreement with our previous studies. KOJI MG84 –our patented molecule- is reported with its cytotoxic activities against A375 human malignant melanoma cells (19). Compared to KOJI MG84, compound **1** showed four times lower cytotoxic activity in the SK-MEL cell line, making it the closest in cytotoxic effect to KOJI MG84. Future experiments will focus on compound 1 and the effect of mechanisms on cell death, which will be tried to identify.

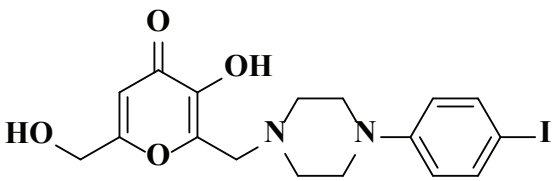

Figure 8. The most effective compound

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Conflict of Interest

The authors have no conflicts of interest.

Statement of Contribution of Researchers

Concept – P.N.U., G.K., C.S.G., M.A.; Design – G.K., M.A.; Supervision – G.K., C.S.G., M.A.; Resources – G.K., M.A.; Materials – G.K., C.S.G., M.A.; Data Collection and/or Processing – G.K., C.S.G., M.A.; Analysis and/or Interpretation – G.K., C.S.G., M.A.; Literature Search – P.N.U. B.D. ; Writing – P.N.U., G.K., B.D.; Critical Reviews – M.A., G.K.

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